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Inhibition by cromoglycate and some flavonoids of nucleoside diphosphate kinase and of exocytosis from permeabilized mast cells

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1 The anti-allergic compound, cromoglycate, is reported to possess affinity for, and to suppress the autophosphorylation of a 72kDa protein having the sequence of nucleoside diphosphate kinase (NDPK).

2 We have tested the ability of cromoglycate, and a panel of ten structurally related flavonoids of plant origin, to inhibit the NDPK reaction and the exocytotic process of permeabilized mast cells. The conditions of permeabilization (use of an isotonic medium based on sodium glutamate) were selected to ensure that NDPK activity would be an essential component in the induction of Ca^{2+}-induced exocytosis in which ATP is required for generation of GTP. For comparison, we also measured the inhibition of exocytosis induced by GTP-γ-S; this proceeds in the absence of ATP and bypasses the need for NDPK activity.

3 We found that cromoglycate does not discriminate between Ca^{2+} and GTP-γ-S-induced exocytosis and is a poor inhibitor of NDPK activity. Concentrations in the millimolar range are required for inhibition of all these functions. By comparison, many of the flavonoids are effective at concentrations in the micromolar range.

4 While we were unable to discern any systematic relationships between their ability to inhibit the three functions, two compounds, quercetin and genistein, inhibit Ca^{2+}-induced, but not GTP-γ-S-induced exocytosis. Inhibition of the late stages of the stimulus-response pathway in mast cells by these compounds is therefore likely to be due to inhibition of NDPK and the consequent failure to generate GTP.

Keywords: Mast cells; exocytosis; cromoglycate; quercetin; flavonoids; calcium; nucleoside diphosphate kinase (NDPK)

Introduction

Understanding of the mechanism and control of exocytosis, the terminal event in the stimulus-secretion pathway, has been advanced by the use of permeabilized cells (Lindau & Gomperts, 1991). By use of such preparations it is possible to control the composition of the intracellular milieu with some degree of precision, and to introduce and test the effects of normally impermeant solutes which might regulate, modulate or inhibit the exocytotic process. The present investigation concerns the mechanism of inhibition by cromoglycate and some structurally related plant flavonoids, of the exocytotic reaction of permeabilized mast cells.

Mast cells permeabilized in buffers formulated with glutamate as the major anion undergo exocytosis in response to non-hydrolysable analogues of GTP (GTP-γ-S, GppNHp etc) (Churcher & Gomperts, 1990) under conditions of very low Ca^{2+} (i.e. less than 10^{-9} M). Alternatively, exocytosis can be induced by Ca^{2+} (buffered at concentrations in the range $10^{-6}-10^{-5}$ M) in the absence of added guanine nucleotide. Unlike the GTP-γ-S-induced response, Ca^{2+} induced exocytosis requires provision of another nucleoside triphosphate such as ATP, UTP etc. (Lillie *et al.*, 1991). Since exocytosis due to Ca^{2+}-*plus*-ATP can be potentiated by addition of GDP at concentrations up to 100 μM (Lillie & Gomperts, 1992a), the role of the nucleoside triphosphate is likely to be that of a phosphate donor in the nucleoside diphosphate kinase (NDPK) reaction. This would allow the formation of GTP from GDP. At concentrations greater than 100 μM, GDP is inhibitory to Ca^{2+}-induced exocytosis. A guanine nucleotide thus appears to be an absolute requirement for exocytosis. Ca^{2+} must be regarded as a modulator, possibly regulating guanine nucleotide exchange on G_E, the GTP-binding protein proposed to mediate exocytosis in these cells (Lillie *et al.*, 1991).

In hepatocyte membranes, NDPK activity has been co-isolated with a GTP-binding protein, G_S, (Kimura & Shimada, 1990) and it has been suggested that it may play a role in the activation of G-proteins through its ability to generate GTP by transphosphorylation (Kimura & Johnson, 1983). We have therefore tested the ability of cromoglycate and some structurally related flavonoids as inhibitors of the NDPK reaction and also as inhibitors of exocytosis from permeabilized mast cells.

Methods

Cell preparation

Mast cells were obtained by peritoneal lavage of large (>300 g) male Sprague Dawley rats. The mast cells were isolated from contaminating cell types by centrifugation through a cushion of Percoll (Pharmacia Ltd, Milton Keynes, Bucks, UK) as previously described (Tatham & Gomperts, 1990). They were washed twice by resuspension and centrifugation.

Secretion assay

The method was as previously described (Tatham & Gomperts, 1990; Gomperts & Tatham, 1992). Cells were suspended at approximately 0.3×10^6 cells ml^{-1} in a buffered electrolyte solution which comprised L-glutamate 125 mM, PIPES 20 mM, Mg PIPES 3 mM, BSA 1 mg ml^{-1}, pH 6.8, 290 mOsm. All salts and reagents were dissolved in this buffer. Cells, streptolysin-O (permeabilising agent, 0.4 iu ml^{-1}) and other re-

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agents, contained in the wells of 96-well microtitreplates were incubated for 10 min at 37°C. Incubations were carried out in 96 'V-well' microtitre plates (Greiner). After incubating the cells for 5 min at 37°C in the presence of metabolic inhibitors (antimycin A, 5 μM and 2-deoxyglucose, 6 mM, to deplete intracellular ATP) samples (about 9000 cells, in 30 μl) were added to wells previously loaded with streptolysin-O, (permeabilising agent, 0.4 iu ml^{-1} final), and either calcium buffers (EGTA, 3 mM final, to regulate Ca^{2+} in the range pCa7-pCa5) with MgATP (1 mM) or GTP-γ-S (at concentrations indicated) with Ca^{2+} buffer (EGTA 3 mM, pCa8). Final volume was 90 μl in each well. Ca^{2+} buffers were prepared as previously described (Gomperts & Tatham, 1992). After incubation at 37°C for 10 min, the reactions were quenched by addition of 100 μl of ice cold buffer and the cells were sedimented by centrifugation (5 min, 400*g*, at 4°C). Samples (50 μl) of supernatant were transferred to the equivalent wells in black plastic, opaque microtitre plates (Dynatech Microfluor) for analysis of secreted β-D-N-acetylglucosaminidase (hexosaminidase). The reactions were initiated by addition of 50 μl of a solution of 4-methylumbelliferyl N-acetyl-β-D glucosaminide (1 mM in Na citrate, 200 mM, pH 4.5, containing Triton X-100, 0.01%). After incubation at 37°C for about 4 h, the reactions were terminated by addition of 300 μl of Tris (0.2 M). Fluorescence (355–460 nm) was measured on a Fluoroskan microtitre plate reader and the readings transferred directly to a computer for manipulation using a standard spreadsheet programme. Calculation of % secretion was based on the comparison of fluorescence measured with control (no cells) and the total cell hexosaminidase content as released by Triton X-100. All secretion reactions were carried out in duplicate, and all experiments were carried out on at least 3 separate occasions.

NDPK assay

The method is based on the measurement of the rate of ADP generation following transphosphorylation from ATP to 2'-dUDP as described in Biochemica Information (Boehringer Mannheim, 1973). The rate of release of ADP is measured as the reduction in the absorbance (340 nm) due to oxidation of NADH as a consequence of reactions catalysed by pyruvate kinase and lactate dehydrogenase in the presence of appropriate substrates and cofactors. All reagents were made up in a solution comprising HEPES (160 mM), MgCl (10 mM) and KCl (10 mM), pH 7.6; 300 μl of a solution containing pyruvate kinase (1.26 iu ml^{-1} final), lactate dehydrogenase (33 iu ml^{-1} final), ATP (6.3 mM final), phosphoenolpyruvate (6.3 mM final), and NADH (1.92 mM final) were placed in wells of a flat-bottomed 96-well microtitre plate. Absorbance measurements were made at 340 nm with a Titertek Multiskan MCC/340 microtitreplate reader. Before starting the reactions, 30 μl 2'-dUDP (final concentration 0.86 mM) was added and a series of absorbance readings were taken (under manual control) to ensure the stability of the system. A range of concentrations of the test compound (10 μl) or buffer (for control) were than added and control of the instrument was transferred to the use of programme MSCAN operated by a PC. This is designed to collect readings from the microtitre plate on a time base and to transfer these as a set of 8 × 12 matrices to a computer file for subsequent analysis on a spreadsheet. Four passes were made to obtain a baseline and then the reactions were initiated by addition with mixing of 10 μl of a suspension of permeabilized mast cells prepared as described in the next paragraph. Alternatively, purified rabbit skeletal muscle NDPK (11.4 iu ml^{-1} final) was used. Measurements were made at room temperature at 15 s intervals.

To prepare mast cells for NDPK measurement, they were first treated with streptolysin-O at ice temperature. This allows the lysin to bind, but not to permeabilize the membrane, and the excess was washed away by centrifugation at 4°C. The cells become permeant as the temperature is elevated to room temperature (i.e. the condition of the enzyme assay). These cells were then used either directly in the NDPK assay to measure total cell activity, or alternatively, they were washed by centrifugation (× 2) at room temperature to remove the cytosolic component.

Presentation of data

Data illustrated in Figures 1, 2 and 3 are taken from single experiments. These are typical of many.

Materials

Nucleoside diphosphate kinase, pyruvate kinase, lactate dehydrogenase, guanosine 5'-[γ-thio]triphosphate (GTP-γ-S), phosphoenol pyruvate and ATP were all obtained from Boehringer Mannheim; 2'-deoxyuridine-5'-diphosphate (dUDP), 4-methylumbelliferyl N-acetyl-β-D-glucosaminide and NADH were obtained from Sigma; 2'-deoxythymidine 5'-diphosphate (dTDP), narigenin, phloretin and phloridzin were obtained from ICN. Samples of purified rutin (quercetin ruti-

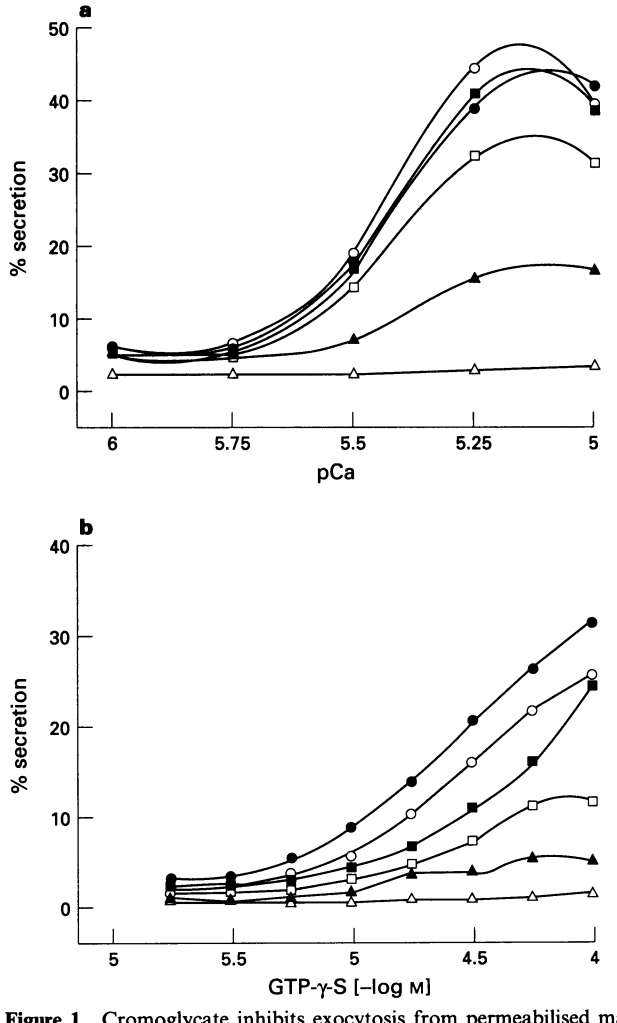

Figure 1 Cromoglycate inhibits exocytosis from permeabilised mast cells: mast cells, suspended in isotonic buffered Na glutamate and pretreated with metabolic inhibitors, were incubated with streptolysin-O, cromoglycate (at the concentrations indicated) and (a) with Ca^{2+} (buffered with EGTA (3mM) in the range pCa6–pCa5) and ATP (1 mM), or (b) with GTP-γ-S (at concentrations indicated) with Ca^{2+} buffered at pCa8. At the end of 10 min the cells were sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase. Cromoglycate concentrations [$-\log_{10}$M]: (a) (●) zero; (○), 4; (■) 3.5; (□) 3; (▲) 2.5; (△) 2; (b) (●) zero; (○) 3.25; (■) 3; (□) 2.75; (▲) 2.5; (△) 2.25.

noside) and acacetin (4'-O-methyl apigenin) were kindly provided by Dr E. Wollenweber (Darmstadt, Germany). Fisetin was obtained from Aldrich. Other flavonoids were purchased from Sigma. Cromoglycate was the gift of Fisons Pharmaceuticals.

Results

Secretion measurements

Secretion was measured from mast cells permeabilized in solutions of isotonic Na glutamate. Under these conditions, exocytosis can be induced either by GTP-γ-S or by Ca^{2+}. For Ca^{2+}-induced secretion it is necessary to provide ATP which is understood to act as a donor in the NDPK reaction for the formation of GTP from GDP (Lillie & Gomperts, 1992a).

Figure 1a illustrates the results of a typical experiment in which we tested the ability of cromoglycate, applied as a range of concentrations ($10^{-4}-10^{-2}$ M) to inhibit exocytosis from permeabilized mast cells stimulated by Ca^{2+}, together with ATP. In agreement with previous work, secretion required Ca^{2+} in the range pCa6–pCa5 and achieved a maximal level of around 50% ($pCa = -\log_{10}[Ca^{2+}]$). Cromoglycate inhibited exocytosis due to the Ca^{2+}-stimulus. Suppression of exocytosis commenced at concentrations greater than $10^{-3.5}$ M and it was capable of causing total suppression at 10^{-2} M with an IC_{50} of 3.18 mM.

Cromoglycate also inhibited exocytosis stimulated by GTP-γ-S as shown in Figure 1b. In this experiment, the concentration of Ca^{2+} was suppressed to 10^{-8} M (pCa8) with EGTA (3 mM). With an IC_{50} of 3.63 mM (see Table 1), the sensitivies of Ca^{2+}-induced and GTP-γ-S-induced secretion to inhibition by cromoglycate were not significantly different ($P>0.75$).

Figure 2 illustrates the effect of the bioflavonoid quercetin on both Ca^{2+}-induced and GTP-γ-S-induced exocytosis from the permeabilized cells. As can be seen in Figure 2a, this compound inhibited secretion due to the Ca^{2+}-stimulus when applied at concentrations in excess of $10^{-5.33}$ M (~ 5 μM) with IC_{50} 54 μM. Quercetin was thus about 100 times more potent than cromoglycate. In contrast, it was without effect on GTP-γ-S-induced secretion when applied at concentrations up to 10^{-4} M (Figure 2b and Table 1).

We tested a panel of 12 structurally related plant flavonoids as possible inhibitors of Ca^{2+}-induced and GTP-γ-S-induced exocytosis. The results are summarised in Table 1 in which the compounds are listed in order of potency as inhibitors for the two systems.

For Ca^{2+}-induced secretion (measured at pCa5), potency appeared to fall into two groups. The first five compounds listed (kaempferol, fisetin, myricetin, phloretin and acacetin) all had IC_{50} below 20 μM. Quercetin, rutin (a glycoside of quercetin), narigenin, genistein and phloridzin (a glycoside of phloretin) had IC_{50}s in the range 50–100 μM. Apigenin and hesperetin are without effect at concentrations less than 100 μM (we were unable to test these two compounds at concentrations higher than this since we found that they interfered with the assay for secreted hexosaminidase). The order of inhibitory potency of Ca^{2+}-independent exocytosis induced by GTP-γ-S (100 μM) by these compounds was different. Here, phloretin, narigenin and acacetin had an IC_{50} below 20 μM with most of the compounds having IC_{50} in the range 20 μM to 60 μM. Apigenin, genistein, hesperetin, and as noted above, quercetin, were without effect on secretion induced by GTP-γ-S.

NDPK activity measurements

We measured the effect of quercetin and the panel of bioflavonoids on the activity of rabbit muscle, and mast cell NDPK. In a preliminary investigation we found that two of the compounds, genistein and myricetin were inhibitory to LDH and we were unable to test these substances as inhibitors of NDPK. An earlier report (Grisolia *et al.*, 1975) indicates inhibition of LDH and pyruvate kinase by quercetin but we were unable to substantiate this, at least in the concentration range relevant to the present work.

Figure 3 illustrates progress curves for ADP generation, catalysed by the NDPK reaction, from permeabilized mast cells. Enzyme activity was associated with both the cytosol and with insoluble structural elements of the cells. The figure illustrates the reaction for the total enzyme content and the residual component remaining after extensive washing of the permeabilised cells; this is likely to represent membrane bound activity (Kimura & Shimada, 1990). The total cell activity of NDPK varied considerably from experiment to experiment, and we have no explanation for this. Nevertheless, our data indicate that a large proportion of the NDPK was retained after washing, and must be presumed to be associated with the cell membranes.

Figure 4 illustrates the inhibition of rabbit muscle NDPK activity by quercetin. The data presented represent the average of six independent experiments in which the initial linear rate of the uninhibited reaction varied between 0.014 and 0.74 absorption units min^{-1}. In two experiments, substitution of 2'-deoxythymidine-diphosphate (2'-dTDP instead of 2'-dUDP) as the substrate for NDPK was without effect on the inhibition due to quercetin (data not shown).

Table 1 Inhibition of exocytosis from permeabilized mast cells by various flavonoids

A *Inhibition of Ca^{2+}-plus-ATP-induced exocytosis*	*Mean IC_{50} (μM)*	*s.e.mean*	n	B *Inhibition of GTP-γ-S-induced exocytosis*	*Mean IC_{50} (μM)*	*s.e.mean*	n
Kaempferol	6.7	1.8	4	Phloretin	6.6	2.2	3
Fisetin	7.4	3.6	4	Acacetin	14.6	1.1	4
Myricetin	8.4	2.2	4	Narigenin	12.5	6.6	4
Phloretin	12.6	7	3	Kaempferol	25.4	8.8	3
Acacetin	15.6	2.8	4	Myricetin	34.2	3.2	4
Quercetin	54.2	11.2	5	Fisetin	43.7	0.5	4
Rutin	65	27	3	Rutin	50.3	13	3
Narigenin	96	14	3	Phlorizin	61.4	25.6	3
Genistein	96	2.9	3	Cromoglycate	3630	1700	4
Phlorizin	103	54.8	3	Apigenin*	No effect		4
Cromoglycate	3180	240	4	Quercetin*	No effect		4
Apigenin	No effect*		4	Genistein*	No effect		3

Statistical analysis using the ANOVA test indicates that the rank orders are significant with $P<0.002$ and $P<0.02$ for Table 1A and 1B respectively.

*These compounds were tested up to a maximum concentration of 100 μM (apigenin and geniestein) and 1 mM (quercetin).

Table 2 lists the panel of flavonoids in the rank orders of their potency as inhibitors of the membrane bound, and the total NDPK activity of rat mast cells. The rank orders of inhibition of the two classes of activity are quite different and neither showed any obvious relationship to the rank orders for inhibition of either Ca^{2+} (plus ATP)-induced exocytosis or GTP-γ-S-induced exocytosis. It is worth noting that genistein, frequently applied to cells as a presumed specific inhibitor of tyrosine kinases (Mustelin *et al.*, 1990), was also an inhibitor of PK and LDH. Cromoglycate was without effect (at concentrations less than 200 μM); because of its absorbance (at 340 nm) at concentrations higher than this could not be tested.

Table 3 lists the flavonoids in their rank order of potency as inhibitors of purified rabbit muscle NDPK. Fisetin, with IC_{50} of 9.3 μM, was the most potent of the compounds tested while cromoglycate at concentrations up to 200 μM was without effect.

Discussion

The main application of cromoglycate is in the prophylactic treatment of bronchial asthma (Foreman & Pearce, 1989). Its mechanism remains poorly defined to the extent that is unknown whether its action on cells is exerted at the cell surface or at an intracellular location. A report showing that it binds and prevents the autophosphorylation of NDPK extracted from RBL-2H3 (rat basophilic leukaemia) cells (Hemmerich *et al.*, 1991; 1992) could be taken to imply an intracellular location for its activity, but its strong acid characteristic ($pK_a = \sim 2$: Cox *et al.*, 1970; Fogg & Fayad, 1978) would argue for impermeability and an action at the cell surface.

The plant flavonoids are compounds having some structural similarity to cromoglycate, most significantly the chroman moeity – two aromatic rings joined by a three carbon unit (C_6-C_3-C_6) (Goodwin & Mercer, 1983). A number of these have been shown to inhibit secretion from mast cells and neu-

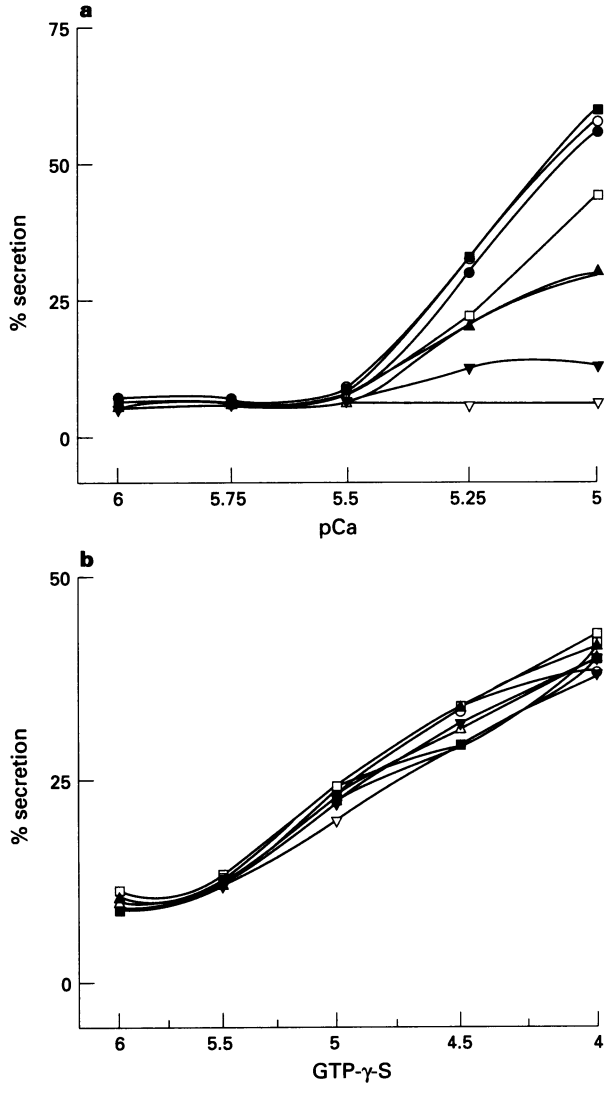

Figure 2 Quercetin inhibits exocytosis from permeabilized mast cells: mast cells, suspended in isotonic buffered Na glutamate and pretreated with metabolic inhibitors, were incubated with streptolysin-O, quercetin (at the concentrations indicated) and (a) with Ca^{2+} (buffered with EGTA (3 mM) in the range pCa6–pCa5) and ATP (1 mM), or (b) with GTP-γ-S (at concentrations indicated) with Ca^{2+} buffered at pCa8. At the end of 10 min the cells were sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase. Quercetin concentrations [$-\log_{10}$M]: (●) zero; (○) 6; (■) 5.67; (□) 5.33; (▲) 4.67; (▼) 4.33; (▽) 4.

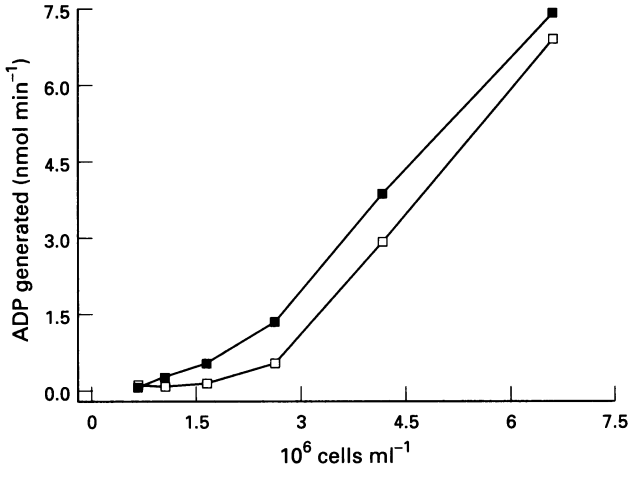

Figure 3 Nucleoside diphosphate kinase activity of unwashed and washed permeabilized mast cells: the rate of ADP generation due to the NDPK reaction of permeabilized mast cells was measured over a range of cell concentrations at room temperature using an enzyme coupled assay. The figure represents the data from a single experiment: (■) whole permeabilized cells; (□) washed permeabilized cells.

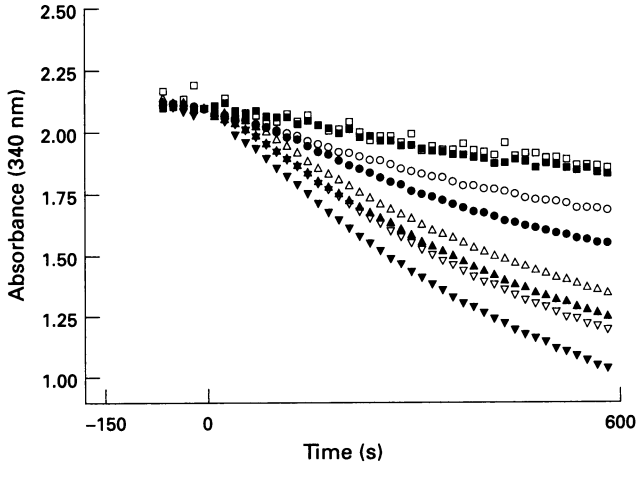

Figure 4 Quercetin inhibits nucleoside diphosphate kinase: the rate of ADP generation as a consequence of rabbit muscle NDPK activity was measured *in vitro* at room temperature using an enzyme coupled assay. The figure represents the normalized data from 6 separate experiments. Quercetin concentrations [$-\log_{10}$M]: (□) zero; (■) 6; (○) 5.67; (●) 5.33; (△) 5; (▲) 4.67; (▽) 4.33; (▼) 4.

Table 2 Inhibition of mast cell nucleoside diphosphate kinase (NDPK) by various flavonoids

	A *Total cell NDPK* Mean IC_{50} (μM)	s.e.mean	n		B *Membrane-bound NDPK* Mean IC_{50} (μM)	s.e.mean	n
Narigenin	3.89	1.87	3	Fisetin	2.83	1.74	3
Rutin	6.89	0.91	3	Quercetin	4.32	1.0	3
Phloretin	9.59	4.41	3	Rutin	6.21	2.89	3
Phlorizin	10.68	3.89	3	Narigenin	13.6	4.82	5
Fisetin	18.05	5.58	3	Morin	13.65	8.46	3
Acacetin	25.61	1.7	3	Phlorizin	16.85	7.99	3
Morin	29.62	0.85	3	Kaempferol	198.7	1.33	3
Quercetin	42.92	10.94	3	Phloretin	>200		4
Kaempferol	No effect*		4	Acacetin	>200		3
Cromoglycate	No effect*		2	Cromoglycate	No effect*		2
Hesperetin	No effect*		3	Hesperetin	No effect*		3

Statistical analysis using the ANOVA test indicates that the rank orders are significant with $P<0.005$.
*These compounds were tested up to a maximum concentration of 200 μM.

Table 3 Inhibition of purified rabbit muscle nucleoside diphosphate kinase (NDPK) by various flavonoids

	IC_{50} (μM)	s.e.mean	n
Fisetin	9.3	2.3	6
Narigenin	9.5	3.9	4
Kaempferol	10.6	2.4	4
Rutin	12.9	3.6	3
Phlorizin	13.2	2.9	5
Phloretin	14.7	2.9	6
Acacetin	20.6	4.3	6
Quercetin	28.6	5.7	6
Cromoglycate	No effect*		4

A statistical analysis using the ANOVA test indicates that the rank order is significant, with $P<0.005$.
*Cromoglycate was tested up to a maximum concentration of 200 μM.

trophils (Fewtrell & Gomperts, 1977a; Bennett *et al.*, 1981) but unlike cromoglycate which had previously been shown to affect ligand-induced secretion (Foreman *et al.*, 1975), these also block secretion due to Ca^{2+}-ionophores (Ennis *et al.*, 1981). Significantly, only the aglycone forms of quercetin and phloretin are inhibitory on intact cells but their glycosides are about equally effective when applied to permeabilized cells (Howell & Gomperts, 1987) suggesting that the flavonoids have an intracellular target for their inhibitory activity. That the actions of cromoglycate and quercetin may be related to a shared target was first suggested by the observation that quercetin is without effect on cells rendered tachyphylactic to cromoglycate (Fewtrell & Gomperts, 1977b). However, while quercetin (and the other flavonoids) clearly affect the exocytotic reaction (this paper and Howell & Gomperts, 1987), cromoglycate suppresses the extent of elevation of Ca^{2+} in antigen-stimulated mast cells, suggesting an additional target upstream of the late stages of signal transduction (White *et al.*, 1984).

In the regulation of secretion from mast cells it has been proposed that Ca^{2+} (with C_E, a Ca^{2+}-binding protein of unknown provenance) and the activating guanine nucleotide (with its associated GTP-binding protein, G_E) act in series. C_E is presumed to act as an exchange catalyst for guanine nucleotides on G_E (Lillie & Gomperts, 1992a,b; 1993). More generally, it has been suggested that the product GTP of the NDPK reaction can be channelled in a manner rendering its interaction with membrane bound G-proteins particularly favourable (Kimura & Shimada, 1988; Wieland & Jakobs, 1992). However, an earlier idea (Otero, 1990; Randazzo *et al.*, 1991) that the NDPK reaction could cause direct phosphorylation of GDP bound to G-proteins, and so set the GTPase cycle in reverse, has been dismissed (Randazzo *et al.*, 1992).

We have examined the effects of cromoglycate and a number of flavonoids in mast cells permeabilized in glutamate-based solutions and stimulated with either GTP-γ-S or Ca^{2+}. Two compounds, quercetin and genistein, are of special interest since they select between the two modes of activation. They inhibit exocytosis due to stimulation by Ca^{2+} (plus ATP) but are without effect on the response due to GTP-γ-S, and therefore probably act only to prevent a reaction linking these two stages of the secretory control pathway. The finding that secretion due to Ca^{2+}-*plus*-ATP is enhanced by GDP (Lillie & Gomperts, 1992a) (also in PC12 cells: Vu & Wagner, 1993) prompted examination of the ability of these compounds (especially quercetin and genistein) to inhibit NDPK.

We have tried to establish whether a correlation exists between the inhibitory activity of these compounds on NDPK and their ability to suppress the stimulation of exocytosis by GTP-γ-S or by Ca^{2+}-*plus*-ATP. Analysis of variance by the ANOVA test indicates that the rank orders presented in the tables (Tables 1,2,3) are significant, with $P<0.02$. Inspection of the various rank orders reveals no simple relationships between the inhibitory functions of these compounds. We have also attempted a more rigorous analysis of the rank orders using the Student Newman-Keuls test (Ostle, 1963) to compare the IC_{50} values of 9 of the flavonoids with each other. The data suggest that there is little, if any relationship between the various inhibitory functions of these compounds.

No attempt has been made here to determine the mechanism of inhibition by any of these compounds for any of the functions investigated, but it is likely that they interfere with the binding of nucleotides. Some of them are known to act as inhibitors of protein kinase C and would therefore be expected to cause inhibition of the ATP-dependent component of secretion (Churcher & Gomperts, 1990) regardless of any effects that they have on NDPK. However, not all of these known inhibitors of PKC inhibit Ca^{2+}-*plus*-ATP induced secretion. More than this, flavonoids are known inhibitors of transport ATPases including Na^+/K^+-ATPases (Kuriki & Racker, 1976; Spector *et al.*, 1980), Ca^{2+}-dependent ATPases (Fewtrell & Gomperts, 1977b; Bennett *et al.*, 1981), and the mitochondrial F_1 ATPase (Carpenedo *et al.*, 1969; Lang & Racker, 1974).

Our observations indicate that cromoglycate inhibits mast cell secretion when provided at concentrations in the millimolar range (see Table 1). Unfortunately, because of its intrinsic absorption at 340 nm we were unable to test its effect on the NDPK reaction at concentrations above 200 μM; at this concentration it was without effect. The IC_{50} for inhibition of autophosphorylation of p72 (NDPK: Hemmerich *et al.*, 1992) isolated from RBL cells is 2 mM (Hemmerich *et al.*, 1991).

In conclusion, since cromoglycate inhibits GTP-γ-S-induced secretion, which occurs in the absence of ATP and therefore with no involvement of NDPK, cromoglycate must act by a mechanism not involving this enzyme. Further, it is improbable

that cromoglycate has an intracellular site of action. In contrast, the flavonoids must penetrate the cells to exert inhibition (Howell & Gomperts, 1987). These compounds are likely to have multiple actions at different points of the stimulus-secretion pathway amongst which are steps close to the exocytotic event itself. At this level the inhibitory effect of cromoglycate must be rated as poor, requiring concentrations in the millimolar range which could never be achieved in intact cells.

Of the flavonoids, quercetin and genistein probably block the terminal stages of secretion by inhibition of NDPK. They inhibit secretion due to Ca^{2+}-*plus*-ATP but not by GTP-γ-S. However, when applied alongside the stimulus to intact cells the onset of the action of quercetin is immediate (Fewtrell & Gomperts, 1977b) and it is improbable that inhibition, e.g. of antigen-induced secretion, could be due to depletion of GTP unless its turnover rate is so rapid that the entire pool is renewed every few seconds. It is more likely that these compounds too, must have other sites of action along the stimulus-secretion pathway. The action of the flavonoid compounds is thus complex, involving a number of intracellular target sites one of which is nucleoside diphosphate kinase.

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